REMARKS

Claims 1-25, 27, 28, 32-57 and 62 are pending. Claims 46-57 have been withdrawn.

CLAIM REJECTIONS

Rejection of claims under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 1-25, 27, 28, 32-45 and 62 under 35 U.S.C. § 112, first paragraph, "as failing to comply with the written description requirement." See Office Action at p. 2. Specifically, the Examiner states that "[t]he specification points to a large number of materials that are capable of being used as shell materials at pages 17-20 of the specification but there is no guidance in the specification to select a shell material that is able to prevent, reduce of inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60[°]C." Id. Applicants respectfully traverse this rejection. Claims 2-25, 27, 28 and 32-45 depend from independent claim 1. Claim 62 is an independent claim.

Claim 1 relates to an antimicrobial material in an encapsulated form that includes (i) a core including an antimicrobial material and (ii) a shell of encapsulating material, wherein the shell of encapsulating material is impermeable to the antimicrobial material and includes a hydrophobic shell material having a melting point above about 45°C, and wherein the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. Claim 62 relates to an antimicrobial material in an encapsulated form, including (i) a core including an antimicrobial material and (ii) a shell of encapsulating material, wherein the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C.

MPEP 2163 (II)(A)(3)(a) states that "[a]n applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics."

As noted by the Examiner, the specification describes a large number of materials that may be used as a shell material. Furthermore, the application sets out that in certain aspects, the shell material should be selected such that it may prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. These teachings must

be read in conjunction with what would be well known to one skilled in the art. The specification cannot be read in simple isolation without this knowledge. These general teachings must also be read in conjunction with the embodiments provided in in the Example section in the specification. In particular, encapsulated nisin is added to sausages prior to heating to an internal temperature of 160°F (see p. 65, lines 4-7 and 17-20 of the specification), to crumpets prior to baking (p. 66, lines 12-13), to processed cheese heated to 60°C, 80°C or 100°C (p. 67, line 27) or bolognaise sauce pasteurized at a core temperature of 80°C (p. 68, line 18). Based on the disclosure above, the skilled person would have readily considered that the inventors had possession of the claimed an antimicrobial material in an encapsulated form described in claims 1 and 62 at the time of filing.

With regard to guidance as to how to identify or select a material able to exhibit these properties described in claims 1 and 62, one skilled in the art would readily understand how to test which of the extensive list of materials provides these properties. The present specification clearly sets out how an antimicrobial material in an encapsulated form in accordance with claim 1 may be prepared. One skilled in the art would understand how to heat such a material to 60°C. One skilled in the art then would fully understand that there are a large number of assays available to identify whether the encapsulated material such as nisin still has the required activity after the 60°C heating. In this respect, Applicants attach a copy of Delves-Broughton et al., "Nisin, natamycin and other commercial fermentates used in food biopreservation," *Woodhead Publishing Limited,* p. 63-99 (2001) at Appendix A ("Delves-Broughton"). Applicants wish to direct the Examiner to Table 3.2 at p. 69 which references a large number of journals in which assays for determining the activity of nisin are disclosed. The listed journals date back to 1964. Thus, Applicants submit that one skilled in the art would very easily ascertain whether an encapsulated material heated to 60°C had prevented, reduced or inhibited heat degradation of the antimicrobial material.

Hence, the specification, taken with the knowledge of a person skilled in the art, sufficiently describes claims 1 and 62 and dependent claims thereof in full, clear, concise and exact terms and satisfies the written description requirement of 35 U.S.C. § 112, first paragraph. Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection of claims under 35 U.S.C. §102(b)

The Examiner has rejected claims 1, 4, 8, 13-16, 18-20, 25, 27-28, 32, 37, 40, 41, 45 and 62 under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 5,204,029 issued to Morgan ("Morgan"). See Office Action at p. 3. Claims 4, 8, 13-16, 18-20, 25, 27-28, 32, 37, 40, 41 and 45 depend from claim 1. Claim 62 is an independent claim.

The Examiner contends that since "[c]laim 25 is a claim that depends from claim 1 which requires the shell materials to be made from fats," "one would expect fats to provide the required properties of the claims." See Office Action at p. 3. The Examiner appears to conclude that any mention in the art of fats, is a disclosure of a material that meets the claim limitation of protecting the antimicrobial material when heated to at least 60°C. However, claims 1 and 62 require that the shell material has a particular property. This property is that it protects the antimicrobial material when heated to a temperature of at least 60°C. Claim 25 is a <u>dependent</u> claim that recites that one class of material which may be used is fats. It may be concluded from this that <u>some</u> fats must have this property. One may not conclude from this that all fats have this property.

MPEP 2131 states that "'[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.' *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987)."

Morgan discloses "[m]icrocapsules having a solid, fusible shell and a multiplicity of liquid cores." See Morgan, Abstract. While Morgan discloses that categories of shell materials can include fats, waxes, and edible materials that can be used to produce foodstuffs (see col. 3, lines 15-21; col. 4, lines 1-3; col. 4, lines 39-41), Morgan **does not disclose either directly or inherently**, selecting a shell material to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. As a result, Morgan does not disclose an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where <u>the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C</u>, as recited in claim 1. Morgan also does not disclose an antimicrobial material in an encapsulated form, including (i) a core including an antimicrobial material and (ii) a shell of encapsulating material, wherein <u>the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C</u>.

Accordingly, Morgan does not anticipate claims 1, 62 and claims dependent therefrom. Applicants respectfully request reconsideration and withdrawal of this rejection.

Berggren

The Examiner has rejected claims 1, 9, 12, 17, 25, 27, 28 and 37 under 35 U.S.C. §102(b) as being anticipated or in the alternative under 35 U.S.C. §103 as being obvious over E.P. Patent Document No. 0687417 A1 ("Berggren"). See Office Action at p. 4. Claims 9, 12, 17, 25, 27, 28 and 37 depend from claim 1.

The Examiner contends that since "[c]laim 25 is a claim that depends from claim 1 which requires the shell material to be made from fats," "one would expect fats to provide the require properties of the claims." See Office Action at p. 5. As the Examiner is aware, claim 25 is dependent on claim 1 and while claim 25 states that the shell material is or can include fats, this must be read in the context of claim 1 which explains that **the shell is selected** to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. As explained above, it may be concluded from this that some fats must have this property. One may not conclude from this that all fats have this property.

Berggren discloses "[a] process for inhibiting pathogenic bacterial growth in chilled cooked meat products which comprises mixing an encapsulated product in particulate form comprising capsules containing acetic acid within an edible lipid with the meat formulation before cooking." See Berggren, Abstract. Berggren further states that "the lipid should be solid at room temperature and have a melting point below the maximum cooking temperature of the meet product." See Berggren, page 2, lines 56-57. Further, the "lipid should not react with meat formulation" and aims "to prevent as much as possible any contact between the acid and the meat until a specific time ...." See Berggren, page 2, lines 32-34 and 57. This is because

Berggren also discloses that the "lipid may be a food grade hydrogenated or partially hydrogenated vegetable or animal fat." See Berggren, page 3, line 8. However, Berggren does not disclose that the lipid is **selected to prevent, reduce or inhibit heat degradation** of the antimicrobial material when heated to a temperature of at least 60°C. As a result, Berggren does not disclose an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C, as recited in claim 1.

Accordingly, Berggren does not anticipate claim 1 and claims dependent therefrom. Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection of claims under 35 U.S.C. §103(a)

Morgan

Claims 1, 4, 8, 9, 11, 13-16, 18-20, 25, 27-28, 32, 37, 39-43 and 45 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Morgan. See Office Action at p. 3. Claims 4, 8, 9, 11, 13-16, 18-20, 25, 27-28, 32, 37, 39-43 and 45 depend from claim 1.

The Examiner contends that since dependent claim 25 "requires the shell material to be made from fats," "one would expect fats to provide the required properties of the claims." See Office Action at p. 3. Applicants respectfully traverse this statement. As explained above, claim 25 is dependent on claim 1 and while claim 25 states that the shell material is or can include fats, this must be read in the context of claim 1 which explains that **the shell is selected** to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. As previously explained, it may be concluded from this that some fats must have this property. One may not conclude from this that all fats have this property. Simply put, if one thinks of a Venn diagram, the class of fats overlaps with the class of shell material protecting antimicrobial material at greater than 60°C. It cannot be deduced that the class of fats falls entirely within the class of shell material protecting antimicrobial material at greater than 60°C.

Morgan teaches "[m]icrocapsules having a solid, fusible shell and a multiplicity of liquid cores." See Morgan, Abstract. Morgan teaches that "[t]he material which is to form the fusible solid shell can, broadly speaking, be any material which can be melted, emulsified, and then solidified." See Morgan, col. 3, lines 11-14. Morgan teaches that characteristics of the shell can include a melting point in the range of 110° F to 195° F, a viscosity similar to the viscosity of the core material at the emulsion temperature, and immiscibility with the core material. See Morgan, col. 4, lines 63-68; col. 6, lines 3-7; col. 6, lines 14-17. Morgan further teaches that categories of shell materials can include fats, waxes, and edible materials that can be used to produce foodstuffs. See Morgan, col. 3, lines 15-21; col. 4, lines 1-3; col. 4, lines 39-41. Morgan does not teach or suggest a shell, where **the shell is selected** to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C.

Morgan does not provide any teaching or motivation to **select** any particular shell material for preventing, reducing or inhibiting heat degradation of antimicrobial material. Nor is

it inherent from the teachings of Morgan that the solid, fusible shell is selected to prevent, reduce or inhibit heat degradation. While Morgan teaches that the cores are surrounded by a shell, this alone does not permit the inference that the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material. See Morgan, col. 3, lines 6-10. Additionally, it is not inherent from the melting points taught by Morgan that the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material. For example, an intact shell, i.e. a shell that has not melted, that allows for increased heat transfer would not prevent, reduce or inhibit heat degradation of the antimicrobial material. As a result, a shell, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C is not inherent from the teachings of Morgan.

Consequently, Morgan does not teach or suggest an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C, as recited in claim 1.

Accordingly, claim 1 and claims that depend therefrom, are patentable over Morgan. Applicants respectfully request reconsideration and withdrawal of this rejection.

Morgan in View of Francis

The Examiner has additionally rejected claims 1-3, 5-10, 21-25, 27, 28, 32, 33, 37 and 45 under 35 U.S.C. §103(a) as being unpatentable over Morgan in view of Francis, *Food Science and Technology*, 2nd edition, Vol. 1, p. 68-69 (2000) ("Francis"). See Office Action at p. 4. Claims 2-3, 5-10, 21-25, 27, 28, 32, 33, 37 and 45 depend from claim 1.

As discussed above, Morgan does not teach or suggest a shell material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. This defect is not remedied by Francis. Francis teaches that compounds, including nisin, have antimicrobial properties. See Francis, pages 68-69. Francis does not teach a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C.

Therefore, neither Morgan nor Francis, alone or in combination teach or suggest an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial

material when heated to a temperature of at least 60°C, as recited in claim 1. Thus, claim 1 and claims that depend therefrom, are patentable over Morgan in view of Francis. Applicants respectfully request reconsideration and withdrawal of this rejection.

Morgan in view of Amankonah

The Examiner has rejected claim 44 under 35 U.S.C. §103(a) as being unpatentable over Morgan in view of U.S. Patent No. 5,516,543 to Amankonah ("Amankonah"). See Office Action at p. 4. Claim 44 depends from claim 1.

As discussed above, Morgan does not teach or suggest a shell material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. This defect is not remedied by Amankonah. Amankonah teaches "oil-coated microparticulated gellan gum microparticles which are useful as a fat replacer, as an encapsulant and/or as a delivery system for food ingredients in low- or no-fat food matrix." See Amankonah, Abstract. Amankonah further teaches that the "[m]icroparticles of the present invention are spherical globules of gellan gum surrounded with an oil coating." See Amankonah, col. 2, lines 27-28. "Any conventional edible oil can be used to prepare microparticles of the present invention." See Amankonah, col. 2, lines 56-57. "Edible fats having relatively high melting points, such as highly unsaturated fats, can be used instead of or in addition to edible oil for coating the gellan gum globule." See Amankonah, col. 2, lines 60-63. However, Amankonah does not teach or suggest selecting an oil to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. As a result, Amankonah does not teach or suggest an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C, as recited in claim 1.

Therefore, neither Morgan nor Amankonah, alone or in combination teach or suggest an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C, as recited in claim 1. Thus, claim 1 and claims that depend therefrom, are patentable over Morgan in view of Francis. Applicants respectfully request reconsideration and withdrawal of this rejection.

claims that depend therefrom, are patentable over Morgan in view of Francis. Applicants respectfully request reconsideration and withdrawal of this rejection.

Berggren

Claims 33-37 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Berggren. See Office Action at p. 5. Claims 33-37 depend from claim 1.

As previously explained, Berggren does not teach that the lipid is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C. Berggren also does not teach or suggest an antimicrobial material in an encapsulated form, including a shell of encapsulating material, where the shell is selected to prevent, reduce or inhibit heat degradation of the antimicrobial material when heated to a temperature of at least 60°C, as recited in claim 1. Accordingly, claims 33-37, which depend from claim 1, are patentable over Morgan. Applicants respectfully request reconsideration and withdrawal of this rejection.

CONCLUSION

Applicant believes that the claims are in condition for allowance. A petition for an extension of time is attached.

Should any fees be required by the present Reply, the Commissioner is hereby authorized to charge Deposit Account 19-4293.

Respectfully submitted,

Date: July 1, 2011

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APPENDIX A

3

Nisin, natamycin and other commercial fermentates used in food biopreservation

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Abstract: The chapter reviews the history, physical and chemical properties, antimicrobial spectrum, mode of action, assay, safety, legislation, and current and potential uses as natural biological preservatives of nisin, natamycin, and undefined fermentates.

Key words: food preservation, natural preservation, spoilage, nisin, Nisaplin®, bacteriocins, natamycin, Natamax®, polyene macrolide, fermentates, MicroGARD®.

3.1 Introduction

Preservatives made by fermentation processes that are available commercially and have approval for use as food additives fall into three categories. These are nisin preparations effective against Gram positive bacteria, particularly spore formers, natamycin preparations effective against yeasts and moulds, and undefined cultured milk and cultured dextrose preparations which, depending on the culture used, can be effective against Gram negative bacteria, Gram positive bacteria, or yeasts and moulds. Use of such preparations made by fermentation is often preferred by food processors as they are considered to be more natural and label friendly methods of food preservation compared to the use of chemicals such as sorbate, benzoates, nitrites and sulphates. This chapter reviews the history, physical and chemical properties, antimicrobial spectrum, modes of action, assay and current and potential applications of nisin, natamycin, and undefined fermentates.

3.2 Nisin used in food biopreservation

Nisin is a polypeptide antibacterial substance or bacteriocin produced by the fermentation of a suitable substrate by certain strains of *Lactococcus lactis* subsp.

lactis (hereafter referred to as *L. lactis)*. Nisin is active against Gram positive bacteria but has little or no effect against Gram negative bacteria, yeasts and moulds.

3.2.1 History

Nisin was discovered in 1928 when inhibitory streptococci were causing problems in the production of cheese due to the inhibition of starter cultures (Rogers, 1928; Rogers and Whittier, 1928). Initially the presence of bacteriophage was suspected but investigations indicated that an inhibitory polypeptide produced by certain strains of *L. lactis* was responsible. Mattick and Hirsch (1947) characterised the compound and called it 'nisin' deriving the name from 'Group **N** **In**hibitory Substance', N being the serotype group determined by the Lancefield serotyping group of streptococci. Early research into nisin and its properties was based on its potential therapeutic effect for veterinary and clinical uses. Due mainly to its relatively narrow antibacterial spectrum, its low solubility in body liquids, and its instability at physiological pH, it has never been developed for such purposes, but interest is still apparent.

Development as a food preservative began in the 1950s. The first report of nisin used as a food preservative was the use of a nisin producing starter to prevent clostridial spoilage of Swiss cheese (Hirsch *et al.*, 1951). McClintock *et al.* (1952) successfully used nisin-producing cultures to inhibit the development of clostridial spores in Gruyere cheese, but problems with inhibition of cheese starter cultures hampered such use (Winkler and Fröhlich, 1957). The development of a dry powder nisin preparation was pioneered by Aplin and Barrett Ltd. in the UK and this resulted in the introduction in 1953 of the first nisin preparation with the trade name of Nisaplin® (Hawley, 1955, 1957). Early uses of nisin were for prevention of clostridial spoilage of processed cheese but since then numerous other applications have been identified and its use is now approved in over 50 countries for a variety of applications (Turtell and Delves-Broughton, 1998). The Nisaplin® product is still in use today, but is now manufactured by Danisco who acquired Aplin and Barrett in 1999. Early preparations were made using a modified milk based medium as substrate and concentrated by foam extraction, but this has now been changed to a sugar-based medium and concentration using membrane technology. The change to a sugar-based medium prevents problems of allergy associated with consumption of dairy products. Other nisin preparations apart from Nisaplin® are now commercially available: brand names include Chrisin® (Chr. Hansens, Denmark), Delvoplus® (DSM, Holland) and Silver Elephant Nisin made by Zheijiang Silver Elephant Bio-Engineering in China. There are also four or five other smaller manufacturers in China. All these preparations have a similar potency and contain 1,000,000 international units (IU) per gram or approximately 2.5% nisin. A difference between Chinese nisin preparations and European produced nisin preparations is that all Chinese preparations are based on nisin Z, whereas nisin manufactured in Europe is all based on nisin A. This difference will be explained later. Due to the fact that all toxicological studies have been carried out on nisin A preparations some countries such as Australia,

New Zealand and Japan specifically state that only nisin A preparations can be used. In other countries that approve the use of nisin either nisin A or nisin Z preparations can be used.

Units of nisin can be confusing. In this chapter nisin concentrations are expressed as levels of pure nisin, i.e. μg/ml or μg/g. Multiplication by 40 will convert these levels to IU (International Unit) /ml or g or level of commercial preparations (mg/kg). For example 1 μg/g of nisin is equivalent to 40 IU nisin/g or 40 mg Nisaplin®/kg.

3.2.2 Physical and chemical properties

Nisin A is a polypeptide consisting of 34 amino acids with a molecular weight of 3510 Daltons. Its unusual structure was solved in 1971 by Gross and Morrell (1971) (Fig. 3.1). It is an atypical protein in that it contains unusual amino acids and lanthionine rings. The presence of lanthionine is now known to be characteristic of a larger group of bacteriocins produced by different Gram positive bacteria and collectively known as 'lantibiotics'. Various natural nisin variants have been discovered. Nisin Z has a substitution of Asn^{27} for His^{27} (Mulders *et al.*, 1991), nisin F has substitutions of Asn^{27} for His^{27} and Val^{30} for $Ileu^{30}$ (de Kwaadsteniet *et al.*, 2008) and nisin Q has substitutions of Val^{15} for Ala^{15}, Leu^{21} for Met^{21}, Asn^{27} for His^{27}, and Val^{30} for $Ileu^{30}$ (Zendo *et al.*, 2003). Nisin potency and spectrum for nisin A and nisin Z are similar, but nisin Z diffuses more readily through agar gel and has a positive charge of 2 compared to a positive charge of 3 for nisin A. Only nisin A and Z are used in commercial preparations. Most published scientific information pertains to nisin A.

Solubility of nisin A is pH dependent (Liu and Hansen, 1990). Thus for pure nisin A solubility at pH 2.2 is approximately 56,000 μg/ml, at pH 5 is 3000 μg/ ml and pH11 is 1000 μg/ml. Solubility is not a problem in food products as nisin levels used are less than 250 μg/ml.

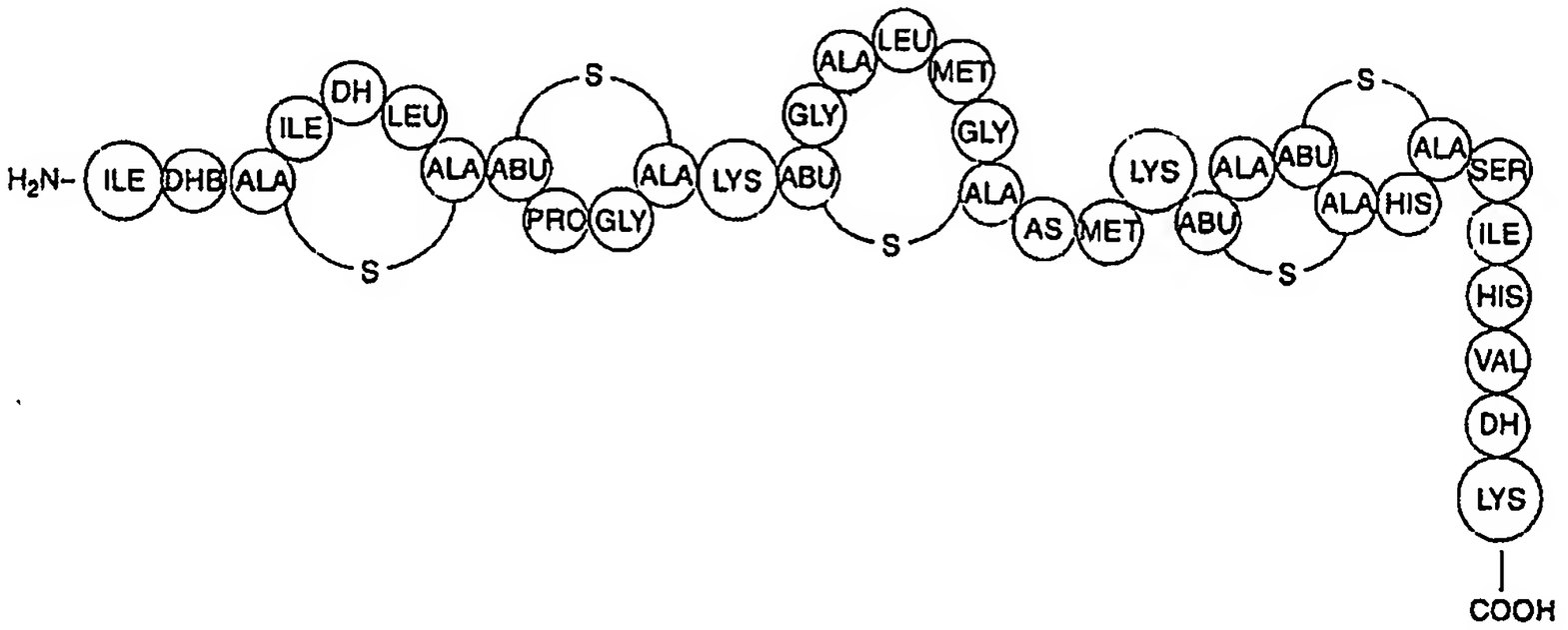

Fig. 3.1 The structure of nisin A. ABA: aminobutryic acid; DHA: dehydroalanine; DHB: dehydrobutyrine (β-methyldehydroalanine); ALA-S-ALA: lanthionine; ABA-S-ALA: β-methyllanthionine.

In the dry state nisin preparations show excellent stability when protected from direct sunlight, moisture uptake, and at temperatures below 22 °C. Nisin A stability of solutions is optimum between pH 3.0 and 3.5. Thus autoclaving nisin A solution in buffer (25 μg/ml) at 121 °C for 15 min resulted in the retention of less than 5% activity at pH1, 42.5% at pH 2, 87.5% at pH 3, 84% at pH 3.5, 80% at pH 4 and 25% at pH 5 (Davies *et al.*, 1998). Even greater losses would be expected at higher pHs near neutrality and above. Pasteurisation temperatures are less damaging to nisin and various components in foods can protect the nisin molecule to an extent from heat.

3.2.3 Antimicrobial spectrum

Nisin has a broad spectrum of activity against Gram positive bacteria and the most significant species associated with food spoilage and safety are shown in Table 3.1. It is important to remember that the sensitivity of nisin to bacteria varies between genera, species and even strains of the same species (Gupta and Prasad, 1989). In normal circumstances nisin does not significantly inhibit Gram negative bacteria, yeasts and moulds.

Among Gram positive bacteria that are sensitive to nisin are members of the mesophilic spore forming genera *Bacillus*, *Alicyclobacillus*, *Clostridium*, *Desulfomaculum*, and the thermophilic spore-forming genera *Geobacillus* and *Themoanaerobacterium*. Both vegetative and spores are sensitive, with levels of nisin required to inhibit spore outgrowth generally less than those required to inhibit vegetative cells. Such an action against spores has resulted in nisin preparations being used as a preservative in products which, by their nature, cannot be fully sterilised but only pasteurised during their production. Nisin also shows activity against many types of lactic acid bacteria. As such bacteria are capable of growth at low pH, nisin can be used as a preservative in low pH foods and beverages that are not heat processed, such as salad dressings, acidified cheese, and alcoholic beverages. The fact that yeasts are insensitive to nisin means that nisin can be used in fermentations alongside yeasts to control the growth of lactic acid bacteria with no effect on the yeast.

3.2.4 Mode of action

Nisin like other preservatives works in a concentration dependent manner in terms of the amount of nisin applied and the level of contamination in the food. Condition of test can dictate whether nisin action against vegetative cells will be predominantly bactericidal or bacteriostatic. The more energised the bacterial cells, the more bactericidal effect the nisin will have, whereas if the cells are in non-energised state because they are in the lag or stationary phase of growth or are in a medium or food of non-optimum pH, water activity, low nutrient availability, and/or at a non-optimum temperature of growth, the nisin effect will be predominantly bacteriostatic (Sahl, 1991; Maisnier-Patin *et al.*, 1995). The use of nisin as a food preservative in combination with other factors is the basis of

Table 3.1 Nisin-sensitive bacterial species associated with food

Genus	Species	Description
Alicyclobacillus	*acidoterrestris*	Heat-resistant spore former. Growth at pH 2.5–6, 25°–60 °C. Spoilage organism of fresh/ pasteurised fruit juice stored at ambient temperature.
Bacillus	*brevis, cereus, coagulans, licheniformis, megaterium, pumilis, subtilis, stearothermophilus*	Heat-resistant aerobic and facultative anaerobic spore formers. Includes psychotrophs, acid-resistant, spoilage organisms, and food poisoning pathogens.
Brochothrix	*thermosphacta*	Heat-sensitive spoilage organism of meat. Growth between 0 °–30 °C. Often associated with modified atmosphere packing.
Clostridium	*bifermentans, botulinum, butyricum, cochlearium, histolyticum, pasteurianum, perfringens, putificum, sordelli, sporogenes, tertium, tyrobutyricum*	Heat-resistant spore-forming obligate anaerobes. Causes spoilage and food poisoning.
Desulfotomaculum	*nigrificans*	Heat-resistant spore-forming obligate anaerobe. Causes blackening of canned food.
Enterococcus	*faecalis, faecium*	Aerobes/anaerobes. Spoilage organism. Varied nisin sensitivity.
Geobacillus	*stearothermophilus*	Thermophilic spore former causes flat-sour spoilage of canned vegetables. Spores very heat resistant.
Lactobacillus	*bulgaricus, brevis, buchneri, casei, curvatus, helveticus, fermentum, lactis, plantarum*	Causes spoilage of acid products, salad dressings, cured meat products, soft drinks, wine, beer, cider. Can grow at low pH.
Leuconostoc	*oenos, mesenteroides*	Aerobes/anaerobes. Causes spoilage of wine and beer. Slime producing.
Listeria	*innocua, monocytogenes*	*L. monocytogenes* – psychroduric food poisoning organism.
Sporolactobacillus	*inulinus*	Aerobe/anaerobe. Spore forming. Growth at low pH.
Staphylococcus	*aureus*	Aerobe/anaerobe. Varied sensitivity. Causes food poisoning.
Thermoanaerobacterium	*thermosaccharolyticum*	Thermophilic spore-former causes can swelling/blowing spoilage of canned vegetables. Spores very heat resistant.

multifactorial preservation otherwise known as 'hurdle technology' (Leistner and Gorris, 1995).

The target for nisin action against vegetative cells is the cytoplasmic membrane. A major breakthrough on the mode of action of nisin against vegetative cells was the discovery that the cell wall peptidoglycan precursor lipid II acts as a docking molecule for nisin, and it is the nisin-lipid II complex that inserts itself into the cytoplasmic membrane forming transient pores that cause leakage of essential cellular material (Breukink *et al.*, 1999; Wiedemann *et al.*, 2001). A further mode of action of nisin is that it also inhibits peptidoglycan synthesis, a component of bacteria cell walls.

The outer membrane of Gram negative bacteria effectively prevents nisin from making contact with the cytoplasmic membrane (Kordel *et al.*, 1989). In combination with a chelating agent such as disodium ethylene-diamine-tetra-acetic acid (EDTA), nisin can be effective against a variety of Gram negative bacteria (Stevens *et al.*, 1991; Delves-Broughton, 1993; Cutters and Siragusa, 1995). Chelating agents remove divalent ions from Gram negative cell walls, releasing phospholipids and lipoproteins thus increasing cell outer membrane permeability. Unfortunately, chelating agents are much less effective in food compared to in buffer solutions due to their preferential binding to free divalent ions within the food. Any treatment such as sub-lethal heat, hydrostatic pressure, pulsed electric field, or freezing which disrupt the outer membrane may render Gram negative bacteria sensitive to nisin.

Mode of action against bacterial spores has not been so intensively studied and it is still uncertain as to its precise mode of action, and even whether it is sporostatic or sporicidal. Thorpe (1960) showed that when nisin was applied to spores of *Geobacillus stearothermophilus*, the reduction in heat resistance observed was apparent rather than real and was due to adsorption of nisin onto the spores and that the nisin could be removed and viability restored if the nisin was removed using the enzyme trypsin. However, more recent research by Gut *et al.* (2008) demonstrated that spores of *B. anthracis* lost their heat resistance when nisin was applied and the spores became hydrated. Previously Morris *et al.* (1984) showed that nisin bound on to sulphydryl groups on protein residues on the spore surface. It is clear that the more spores are heat damaged the more they are sensitive to nisin and that thermophilic spores belonging to *Geobacillus stearothermophilus* and *Thermoanaerobacterium thermosaccharolyticum* are extremely sensitive.

3.2.5 Assay

Basically nisin can be measured in two ways – either directly by chemical, immunological, or genetic measurement of the nisin molecule; or indirectly by measuring its biological activity or potency by turbidometry, agar diffusion assay, or measurement of efflux of cellular material. The various methods with their limit of detection are shown in Table 3.2. The preferred method of quantitative assay of nisin in foods is the *Micrococcus luteus* plate diffusion assay.

Table 3.2 Methods for assay of nisin with approximate minimum levels of detection

Method	Detection limit	Reference
Biological activity measurement		
Turbidometric assay	0.025 μg/ml	Barreteau *et al.* (2004), Turcotte *et al.* (2004)
Agar diffusion assay	0.025 μg/ml	Tramer and Fowler (1964), Fowler *et al.* (1975)
Efflux and assay of adenosine triphosphate	0.025 μg/ml	Waites and Ogden (1987), Valat *et al.* (2003)
Efflux and assay of potassium	0.018 μg/ml	White *et al.* (1992), Mugochi *et al.* (2001)
Impedance	0.05 μg/ml	Giraffa *et al.* (1990), Čurda *et al.* (1995), Kozáková *et al.* (2005)
Chemical measurement		
High performance liquid chromatography	0.25 μg/ml	Delves-Broughton and Friis (1998), Matusaki *et al.* (1995)
Enzyme linked absorption assay	5–10 ng/ml	Falahee *et al.* (1990), Leung *et al.* (2002), Suárez *et al.* (1996)
Genetic-based bioluminescence	0.0125–0.75 ng/ml	Wahlström and Sarris (1999), Reuanen and Saris (2003)
	0.02–10 pg/ml	Hakovirta *et al.* (2006), Hanan *et al.* (2009)

3.2.6 Current applications of nisin in foods

Use of nisin in foods is dependent on regulatory approval which varies from country to country. Nisin is often used as a preservative in foods which are pasteurised but not fully sterilised during production thus protecting the food from outgrowth of spores which survive the pasteurisation process. Nisin insensitive organisms such as Gram negative bacteria, yeasts, and moulds are sensitive to heat and will be killed by the pasteurisation. Nisin can also be used to control contaminant lactic acid bacteria in the brewing and wine making process where its lack of effect against yeasts is a benefit. Applications are shown in Table 3.3.

The outcome of nisin activity within a food system will depend on numerous factors. Other preservative hurdles such as severity of heat treatment, low water activity, modified atmosphere, low temperature, low pH, and the presence of other natural or chemical preservatives can enhance activity. Nisin works better in liquid or homogenous foods compared to solid or heterogenous products because the bacteriocin can be better or more evenly distributed in the former. Nisin is hydrophobic in nature so fat in food may hinder its distribution or render it unavailable for activity (Jung *et al.*, 1992). Certain food additives have been

Table 3.3 Examples of nisin applications, typical addition levels, and supporting references

Food	Nisin (μg/g)	Typical target organism	Reference
Processed cheese	2.5–15	*Bacillus, Clostridium*	Somers and Taylor, (1987), Delves-Broughton (1998)
Milk and milk products	0.25–1.25	*Bacillus, Clostridium*	Maisnier-Patin *et al.* (1995), Wirjantaro and Lewis (1996), Wirjantaro *et al.* (2001)
Pasteurised chilled dairy desserts	1.88–5	*Bacillus, Clostridium*	Sukumar *et al.* (1976), Anonymous (1985)
Liquid egg	1.25–5	*Bacillus*	Delves-Broughton *et al.* (1992)
Pasteurised soups	2.5–6.25	*Bacillus*	
Crumpets	3.75–6.25	*Bacillus cereus*	Jenson *et al.* (1994)
Fruit juice	0.75–1.5	*Alicyclobacillus acidoterrestris*	Komitopoulou *et al.* (1999), Yamazaki *et al.* (2000), Peña and de Massaguer (2006), Walker and Phillips (2008)
Canned food	2.5–5	*Geobacillus stearothermophilus, Thermoanaerobacterium thermosaccharolyticum*	Gillespy (1953), O'Brien *et al.* (1956), Duran *et al.* (1964), Hernandez *et al.* (1964), Nekhotenova (1961), Vas *et al.* (1967)
Dressings and sauces	1.25–5	Lactic acid bacteria, *Bacillus*	Muriana and Kanach (1995), Beuchat *et al.* (1997)
Processed meats such as bologna, frankfurter sausages	5–10	Lactic acid bacteria, *Brocothrix thermosphacta*	Davies *et al.* (1999), Gill and Holley (2000)
Ricotta cheese	2.5–5	*L. monocytogenes, Bacillus*	Davies *et al.* (1997)
Beer		Lactic acid bacteria	
Reduced pasteurisation	0.25–1.25		Ogden (1986), Ogden *et al.* (1988)
During fermentation	0.63–2.5		
Post fermentation	0.25–1.25		

shown in our laboratories to be antagonistic to nisin and these include sodium metabisulphite (antioxidant, bleaching agent and broad spectrum preservative) and titanium dioxide (whitener). In foods that are not heat treated, or that have been minimally processed, nisin may be degraded by proteolytic enzymes.

During heat processing a certain amount of the nisin will be degraded. This will depend on the severity of the heat treatment, pH of the food, and the degree of protection the food may give the nisin. For example, in processed cheese manufacture about 20–25% can be lost during a typical melt process; and in retorting of canning of vegetables where nisin is used to protect against thermophilic spoilage, up to 95% can be lost. The low residual levels in canned vegetables are still very effective reflecting the extreme sensitivity of thermophilic spore formers to nisin. Similarly, nisin retention in foods will be dependent on the food itself, pH and the length and temperature of storage. Low pH is beneficial on two counts: first, nisin retention during heat processing is optimum at low pH; and second, low pH is often a further hurdle in itself inhibiting bacterial growth.

The use of nisin in beer and wine production makes use of the fact that nisin has no effect on yeast viability and vitality but is active against many of the Gram positive lactic acid bacteria that can spoil beer and wine. Uses of nisin in beer especially for washing pitching yeast have been proposed (Ogden, 1986, 1987; Ogden and Tubb, 1995; Ogden *et al.*, 1988). Uses of nisin in wine have also been proposed (Radler, 1990a, 1990b; Daeschel *et al.*, 1991; Knoll *et al.*, 2008). Nisin may also have potential in fuel alcohol production by inhibiting lactic acid bacteria competing with yeast for substrate (Mawson and Costar, 1993; Franchi *et al.*, 2003a, b).

3.2.7 Potential applications of nisin in foods

New combinations of nisin with other preservatives

The use of nisin in combination with other preservatives and food ingredients with the objective of finding combinations that demonstrate additive or synergistic effect has been the subject of much research and many successful combinations have been identified. Space does not allow all to be described, but some examples are shown in Table 3.4. Synergies usually occur with nisin in combination with other preservatives that have the cytoplasmic membrane as target (Adams and Smid, 1983). Much of the research has been carried out with *L. monocytogenes* as the chosen target bacteria which reflects the concern in the USA to the problem of listeriosis and their zero tolerance policy on the presence of the pathogen in foods that are not heated sufficiently to kill the bacteria prior to consumption. With the increased development of chilled long-shelf-life, ready to eat meals, concern is now being directed at the need to ensure against botulism. Powerful synergies that both increase the effectiveness and broaden the antimicrobial spectrum of nisin may be required to be subjected to toxicological review to ensure they are safe.

Nisin in combination with novel food processing technology

Increasing consumer demand for minimally processed, shelf-stable foods has prompted food technologists and scientists to explore other physical preservation

Table 3.4 Examples of published papers demonstrating nisin synergy with other antimicrobials

Antimicrobial substance	Target organism(s)	Substrate	Reference
Organic acids			
Potassium sorbate	*L. monocytogenes*	CO_2 and vacuum packed beef	Avery and Buncic (1997)
Acetic acid	*E. coli*	Ground beef	Fang and Hseuh (2000)
Potassium sorbate Sodium benzoate	*S. aureus B. cereus*	Vegetarian food	Fang *et al.* (1997)
Sodium lactate	*L. monocytogenes*	Vacuum packed smoked salmon	Neetoo *et al.* (2008)
Monoglycerides			
Monolaurin	Spoilage bacteria	Model meat system	Bell and de Lacy (1987)
Monolaurin	*L. lactis* subsp. *agalactiae*	Milk	Blackburn *et al.* (1989)
Sucrose fatty acid esters			
Sucrose palmitate Sucrose stearate	Various Gram positive bacteria	Buffer and agar medium	Thomas *et al.* (1998)
Chelating agents			
EDTA	Gram negative bacteria	Buffer	Stevens *et al.* (1992a,b)
EDTA	*L. monocytogenes*	Vacuum packed beef	Zhang and Mustapha (1999)
EDTA	*Pseudomonas*	Whole and cut melon	Ukuku and Fett (2002)
Maltol	*E. coli*	Buffer	Shved *et al.* (1996)
Lactoperoxidase system			
	L. monocytogenes	Skimmed milk	Zapico *et al.* (1998)
	L. monocytogenes	Milk	Boussouel *et al.* (1999, 2000)
	Various bacteria	Sardines	Elotmani and Assobhei (2003)
Lysozyme			
	Listeria spp.	Processed cheese, paté	Ter Steeg (1993)
	L. monocytogenes, *S. aureus*	Hot dogs	Proctor and Cunningham (1993)
	Various lactic acid bacteria	Broth	Chung and Hancock (2000)
Other bacteriocins			
Pediocin	Various Gram positive bacteria	Buffer	Hanlin *et al.* (1993)
Pediocin ACH, Lacticin 481	Various Gram positive bacteria	Broth and agar medium	Mulet-Powell *et al.* (1998)
ε-Poly-L-lysine			
	L. monocytogenes, *Bacillus*	Buffer	Najar *et al.* (2007)

Reuterin			
	L. monocytogenes, *S. aureus*	Milk	Arqués *et al.* (2008)
Lactoferrin			
	L. monocytogenes	Broth	Cleveland and Tchikindas (2001)
	Bacterial flora	Meat balls	Colak *et al.* (2008)
Essential oils			
Cavacrol	*B. cereus*, *B. circulans*	Broth, potato puree	Rajkovic *et al.* (2005)
Grape seed extract, Green tea extract	*L. monocytogenes*	Soy protein film on frankfurters	Theivendran *et al.* (2006)
Rosemary extract	*L. monocytogenes*, *B. cereus*	Bolognese sauce, carbonara sauce	Thomas and Isak (2006)

methods as alternatives to traditional treatments such as freezing, canning, or drying. Although these traditional technologies have helped to ensure a high level of safety, the heating and cooling of foods can contribute to deterioration of various quality attributes such as colour, nutritional content and flavour (Delves-Broughton, 2008). Promising novel methods of preservation of food and beverages include the use of ultra high pressure (UHP), pulsed electric field (PEF), edible coatings and active packaging. Nisin as an adjunct to all four of these novel methods of preservation has been the subject of considerable research.

Nisin in combination with UHP

UHP shows considerable promise as a novel means of food preservation and a number of commercial foods are now processed using the technology (Black *et al.*, 2005; Yaldagard *et al.*, 2008). Examples are ready-to-eat chicken meat, sliced ham, fresh whole oysters, jams, fruit juices and guacamole. Methods and equipment used for UHP treatment are outlined in review articles by Cheftel (1995) and Yaldagard *et al.* (2008). The commercial success of UHP will depend upon the effective destruction and/or control of food pathogenic and spoilage microorganisms. The vegetative cells of bacteria, moulds and yeasts and spores of moulds can be reduced by 6 log cycles at or below 690 Megapascals (MPa) at ambient temperature or by a combination of less pressure but increased temperature. Bacterial spores are far more resistant and a 5–6 log reduction of *Bacillus* and *Clostridium* spores requires a combination of very high pressure and high temperature (Farkas and Hoover, 2000; Ray *et al.*, 2001). Such a drastic treatment can be costly in terms of equipment design and operating costs (Gao and Ju, 2008) and may adversely effect the quality of many foods. This has prompted the evaluation of nisin as an adjunct to UHP treatment of foods as a means of reducing the level of pressure required to ensure required shelf life and safety.

The mode of action of UHP against microorganisms is that it causes aggregation of proteins and disruption of the cytoplasmic membrane (Rovere *et al.*, 1998). It

has been demonstrated that nisin and UHP not only are synergistic in the killing of Gram positive bacteria but can also widen the spectrum to kill Gram negative bacteria and to a lesser extent yeasts. The sensitisation of Gram negative bacteria by UHP to nisin is considered to be due to outer membrane damage allowing nisin to reach the target site, the cytoplasmic membrane (Kalachayanand *et al.*, 1994; Hauben *et al.*, 1996; Masschalck *et al.*, 2001; Black *et al.*, 2008).

An alternative approach to destruction of spores using UHP is to to induce germination by low to medium range hydrostatic pressure to cause germination and outgrowth and then expose the outgrown vegetative cells to nisin, thus preventing their multiplication (Stewart *et al.*, 2000; Kalachayand *et al.*, 2004).

Nisin in combination with PEF

The mode of action of PEF is that it produces structural changes in the cytoplasmic membrane resulting in pore formation, efflux of essential cellular material and a loss of selective permeability. Since nisin and PEF both act on cytoplasmic membranes it is logical to predict that their combination or use in sequence would have additive or synergistic bactericidal effects. PEF treatment of foods is restricted to pumpable liquid foods and research has been carried out in milk, soups, fruit juice and liquid egg products. Detailed information on PEF theory, and equipment and technology, can be found in a review by Vega-Mecardo *et al.* (1999).

Several investigations have demonstrated that nisin in combination with PEF is effective in buffer and various liquid foods and beverages (Table 3.5). Exposure of *Listeria innocua* to nisin in liquid whole egg following PEF treatment exhibited an additive effect on the inactivation of the bacteria (Calderón-Miranda *et al.*, 1999a). A synergistic effect was observed as the electric field intensity (30–50 kV/cm), number of pulses and nisin concentration (0.25–2.5 μg/ml) increased both in liquid egg and in skimmed milk (Calderón-Miranda *et al.*, 1999a,b). Transmission electron microscopy reveals that *L. innocua* treated by PEF alone in skimmed milk exhibited an increase in the cell wall roughness, cytoplasmic clumping, leakage of cellular material, and rupture of cell walls and cell membranes, whereas treatment with nisin and PEF in combination exhibited an increase in cell wall width (Calderón-Miranda *et al.*, 1999c). Thus the combination may cause damage of the cell wall rather than the cell membrane. Interestingly it has been observed that the efficiency of a combined treatment of nisin and PEF in liquid whey protein was strongly dependent on the sequence of application, since exposure to nisin after PEF produced a lower effect on *L. innocua* inactivation.

Studies have demonstrated the efficacy of PEF against Gram negative bacteria can be enhanced by nisin. When PEF treatment was applied to *Salmonella* cells in the presence of nisin (2.5 μg/ml), lysozyme (2,400 units/ml) or a mixture of nisin (0.688 μg/ml) and lysozyme (690 units/ml), cell inactivation by the combination was increased by an additional 0.04 to 2.75 log units. Furthermore, the combination of nisin and lysozyme had a more pronounced bactericidal effect (by at least 1.37 log cycles) than either nisin or lysozyme alone (Liang *et al.*, 2006).

Table 3.5 Reported effects of the application of nisin and pulsed electric field for microbial inactivation

References	Observed effects
Dutreux *et al.* (2000)	Increased activation of *M. luteus* in phosphate buffer
Santi *et al.* (2003)	Increased activation of *P. aeruginosa*
Sobrino-López and Martín-Belloso (2006, 2008)	Increased activation of *S. aureus* in skimmed milk
Calderón- Miranda *et al.* (1999 a,b,c), Gallo *et al.* (2007), Miranda *et al.* (2001)	Increased inactivation of *L. innocua* in liquid whole egg, skimmed milk, and liquid protein concentrate
Terebiznik *et al.* (2000, 2001, 2002)	Increased inactivation of *E. coli* in simulated milk ultrafiltrate media Observed synergism with reduced water activity
Pol *et al.* (2000, 2001a,b)	Increased inactivation of *B. cereus* vegetative cells (more efficient in buffer than skimmed milk) Observed synergism with cavacrol
Liang *et al.* (2006)	Inactivation of *Salmonella* in orange juice. Observed synergism with lysozyme
Hodgins *et al.* (2002)	Increased inactivation of microorganisms in orange juice. Observed synergism with lysozyme
Iu *et al.* (2001)	Inactivation of *E. coli* 0157:H7 in fresh apple cider. Observed synergism with cinnamon
Ulmer *et al.* (2002)	Inactivation of *L. plantarum* in model beer
Nguyen and Mittal, (2007)	Increased inactivation of microorganisms in tomato juice

Modified and expanded from Gálvez *et al.* (2007).

It should be noted that bacterial spores are resistant to PEF treatments. Incorporation of nisin into food may provide an additional hurdle if the nisin survived the PEF treatment against surviving spores. However, limited evidence to date suggests that nisin is destroyed by PEF treatment (Terebiznik *et al.*, 2000). To make use of nisin as a means of preventing spore outgrowth, the nisin would have to be added aseptically to the food post PEF treatment or possibly protected during the PEF treatment by encapsulation.

Use of nisin in active antimicrobial packaging

Antimicrobial active packaging acts by inhibiting or killing the growth of undesirable microorganisms on the surface of foods. Of all the antimicrobials studied for their effectiveness in both edible and non-edible films, nisin has been the most extensively studied. It has been studied alone or in combination with other antimicrobial agents such as EDTA, lysozyme, organic acids, grape seed extract and green tea extract. Joeger (2007) carried out an extensive review of the literature and found that the majority of results reported around a log 2

reduction of target vegetative cells although at times it was significantly higher. Most studies use as test organism *L. monocytogenes* which again reflects the concern for this psychroduric pathogen, particularly in the USA. Joeger concludes that active antimicrobial packaging still faces limitations and is best viewed as a part of a hurdle strategy to provide safe foods or as a method of increasing shelf life.

3.2.8 Safety and tolerance

Nisin-producing *L. lactis* occur not only in raw cow's milk and cheese but have been found in a variety of other foods and even human breast milk (Table 3.6). Inadvertently and apparently harmlessly, humans and animals probably have consumed nisin, albeit in small amounts, for centuries. Numerous toxicological studies have been carried out and it should be noted that all these have been confined to nisin A preparation. No toxicological study has been carried out with

Table 3.6 Foods and other sources from which nisin producing *L. lactis* have been isolated

Food or other sources	Reference
Cow's milk	Rogers (1928), Rogers and Whittier (1928), Delves–Broughton (1990), Rodríguez *et al.* (2000), Şanilibaba *et al.* (2009)
Bovine milk, Grana cheese	Carini and Baldini (1969)
Sauerkraut (fermented cabbage)	Harris *et al.* (1992), Tolonen *et al.* (2004)
Mixed salad, fermented carrots	Uhlman *et al.* (1992)
Buffalo market milk	Gupta *et al.* (1993)
Various cheese, bovine milk, and meats	Vaughan *et al.* (1994)
Dry fermented sausages	Rodríguez *et al.* (1995)
Various ready to eat meats, fish, cheeses, vegetables	Kelly *et al.* (1996, 1998)
Soil, effluent water, cattle skin	Klijn *et al.* (1995)
Bean sprouts	Cai *et al.* (1997)
Kimchi (fermented cabbage)	Choi *et al.* (2000)
Bovine milk, goat milk, Chinese radish seed, soil, saliva of cow	Ayad *et al.* (2002)
River water	Zendo *et al.* (2003)
Human breast milk	Beasley and Saris (2004)
Rigouta cheese (Tunisia)	Ghrairi *et al.* (2004)
Freshwater catfish	De Kwaadsteniet *et al.* (2008)
Tsuda–turnip pickles	Aso *et al.* (2008)
Tunisian cheeses	Ouzari *et al.* (2008)
Slovenian cheese	Trmčič *et al.* (2008)

nisin Z or any other nisin variant. The studies carried out with nisin A preparation confirm that nisin A is non toxic at levels much higher than those used in food (Frazer *et al.*, 1962; Hara *et al.*, 1962; Bogorditskaya *et al.*, 1970; Shtenberg and Igant'ev, 1970). Digestive enzymes rapidly inactivate nisin and consequently do not alter the microflora in the intestinal tract (Barber *et al.*, 1952; Heinemann and Williams, 1966; Jarvis and Mahoney, 1969). The LD_{50} value is about 7g/kg body weight, similar to that of common salt. As the preparation tested contained 75% salt, the toxicity can be attributed to that component alone (Hara *et al.*, 1962). No ill effects were observed in pigs and poultry from feeding experiments (Barber *et al.*, 1952; Coates *et al.*, 1951). There is no evidence of any cross resistance with antibiotics used in medicine (Szybalski, 1953; Carlson and Bauer, 1957; Hossack *et al.*, 1983; Chikindas *et al.*, 2000).

In 1969 the FAO/WHO Expert Committee decided from the available evidence that a suitable acceptable daily intake (ADI) was 33,000 IU (0.825 mg nisin A)/kg of body weight/day. In 1988, the US Food and Drug Administration (FDA) affirmed nisin as GRAS (generally recognised as safe) for direct use as a food ingredient (FDA, 1988). The EU Expert Scientific Panel (EFSA) reviewed nisin as a food additive in 2006 and concluded that it was a safe and useful preservative (EFSA, 2006). Various expert opinions outline the reasons as to how nisin is different from antibiotics and to why it is a safe food preservative and should be considered for wider use (Hurst, 1981; Wessels *et al.*, 1998; Cleveland *et al.*, 2001).

3.3 Natamycin used in food biopreservation

Natamycin, previously sometimes known as pimaracin or tennectin, is a polyene macrolide antimycotic produced by the actinomycete *Streptomyces natalensis* and other closely related *Streptomyces* spp. Natamycin is active against yeasts and moulds, and shows no activity against bacteria.

3.3.1 History

Natamycin was first produced in 1955 from a culture filtrate of a Streptomycetes isolated from a soil sample in South Africa (Struyk *et al.*, 1959; Brik, 1981).

It is produced by fermentation of *S. natalensis* in a medium containing a carbon source (e.g., starch or molasses) and a fermentable nitrogen source (e.g., corn steep liquor, casein, soya bean meal). Fermentation is aerobic and mechanical agitation and antifoaming agents can aid the process. The temperature range is 26–30°C and the pH range is 6–8. Due to its low solubility, natamycin will accumulate mainly as crystals and these can be extracted following separation of the biomass by solvent extraction (Struyk and Waivisz, 1975).

Natamycin preparations have been used for several years as a preservative protecting foods and beverages against yeast and mould spoilage. Many applications are in bacteria fermented foods prone to yeast or mould spoilage as

the preservative has a selective action against yeasts and moulds with no action against bacteria. Commercial preparations available are Natamax® (Danisco, Denmark), Delvocid® (DSM, Holland) and Silver Elephant Natamycin (Zheijiang Silver Elephant Bio-Engineering, China). The natamycin content of most preparations is 50% with the incipient being lactose, glucose, or salt. Preparations are also available that contain food grade polymers that aid the adherence of natamycin for surface treatments of foods (Delves-Broughton *et al.*, 2006).

3.3.2 Physical and chemical properties

Natamycin belongs to a group of antifungals known as polyene macrolides. The structure (Fig. 3.2) was first determined by Ceder (1964) and the stereo structure by Lancelin and Beau (1995). It has a molecular weight of 665.7 Daltons, is amphoteric and has an isoelectric point of 6.5. Natamycin is a white/cream-coloured crystalline powder with no taste and little odour. It is stable in powder form if stored at room temperature but in aqueous solutions is less stable particularly if exposed to acidic conditions, light, certain oxidants and heavy metals (Raab, 1972). Natamycin has low solubility in water (approximately 40 μg/ml), but this low solubility is an advantage in the surface treatment of foods because it ensures that the preservative remains on the surface of the food where it is needed, rather than migrating into the foods. Increased solubility occurs with a range of solvents (Delves-Broughton *et al.*, 2005).

Raab (1972) reports on the effect of pH on stability of natamycin solutions: it is more stable in the pH range 4.5 to 9, and at pHs above and below this it is significantly less stable.

3.3.3 Antimicrobial spectrum

Natamycin is effective against a wide range of yeasts and moulds and the preservative is usually effective at concentrations between 1 and 10 μg/ml. In

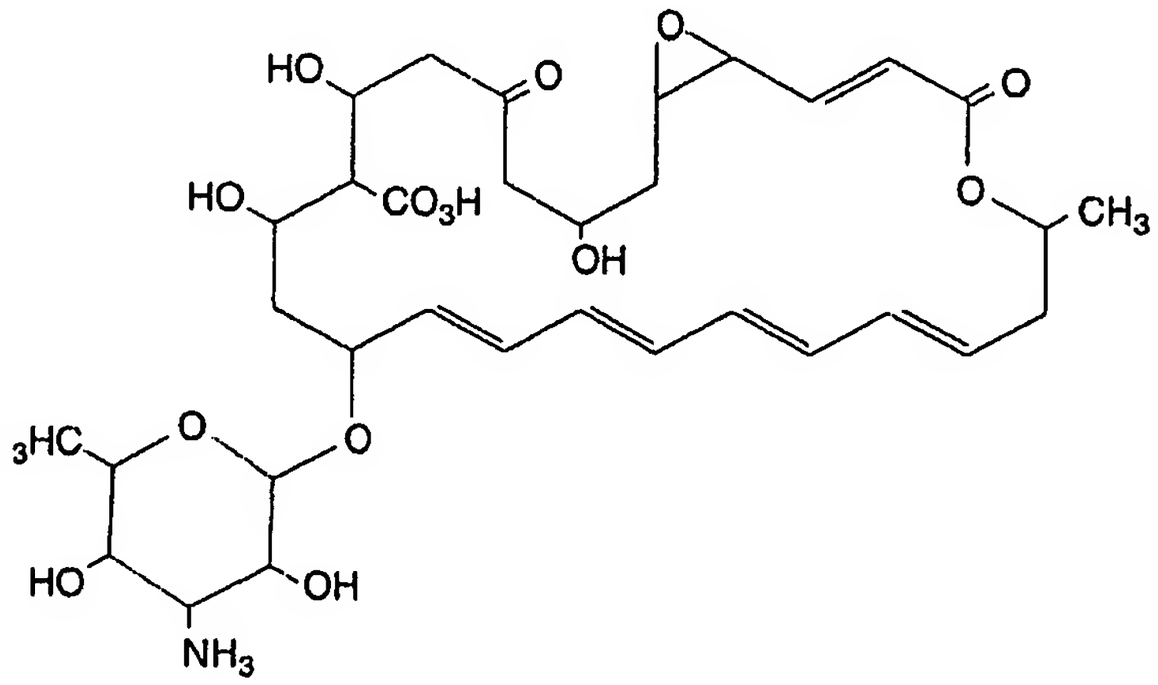

Fig. 3.2 The structure of natamycin.

general yeasts are more sensitive than moulds, the minimum inhibitory concentrations (MIC) of yeasts are usually less than 5 μg/ml whereas that of moulds can be 10 μg/ml or higher. Examples of yeasts and moulds sensitive to natamycin are shown in Table 3.7.

3.3.4 Mode of action

The mode of action of natamycin involves an interaction between natamycin and ergosterol, an essential component of membranes of yeasts and moulds. Originally it was proposed that this interaction resulted in increased membrane permeability efflux of cellular material. However, recent research by Te Welscher *et al.* (2008) and van Leeuwen *et al.* (2009) has shown that the action of natamycin does not increase permeability of the cytoplasmic but more likely prevents cell growth, spore germination, and inhibits membrane associated enzyme activity. *Penicillium discolor*, *Verticillium cinnabarinum* and *Botrytis cinerea*, three moulds with reduced ergosterol content in their cell membrane and ergosterol deficient mutants of *Aspergillus nidulans*, have much reduced natamycin sensitivity (Ziogas *et al.*, 1993). De Boer and Stolk-Horsthuis (1977) and De Boer *et al.* (1979) compared the sensitivity of yeasts and moulds from cheese and sausage factories where natamycin had been used for several years and where it had never been used.

Table 3.7 Examples of yeasts and moulds that are sensitive to natamycin

Absidia	*P. commune*
Alternaria	*P. chysogenum*
Aspergillus chevalieri	*P. cyclopodium*
A. flavus	*P. digitatum*
A. niger	*P. expansum*
A. ochraceus	*P. islandicum*
A. oryzae	*P. notatum*
A. penicilloides	*P. roqueforti*
A. roqueforti	*Rhizopus oryzae*
A. versicolor	*Rhodotolura gracilis*
Botrytis cinerea	*Saccharomyces bailii*
Brettanomyces bruxellensis	*S. bayanus*
Bassochlymas fulva	*S. cerevisiae*
Candica albicans	*S. exiguus*
C. guillermondii	*S. florentimus*
C. vini	*S. ludwigii*
Cladosporium cladosporiodes	*S. rouxii*
Fusarium	*S. sake*
Gloeosporium album	*Sclerotina fructicola*
Hansenula polymorpha	*Scopulariopsis saperula*
Koeckera apiculata	*Toluropsis candida*
Mucor mucedo	*T. lactis-condensi*
M. raceosus	*Wallensis sebii*
Penicillium camemberti	*Zygosaccharomyces barkeri*

There was no difference in the sensitivity to natamycin of yeasts and moulds between sites.

3.3.5 Method of assay

Shirk *et al.* (1962) developed an agar diffusion bioassay using *Saccharomyces cerevisiae* as indicator organism. HPLC is however the preferred method of assay (Anon., 2008). Surface natamycin can be extracted from the surface of foods using methanol and the limit of detection for the HPLC assay is 0.5 μg/g. Various other methods have been described, such as ultraviolet spectrophotometry (Capitán-Vallvey *et al.*, 2000) and enzyme immunoassay (Maertlbauer *et al.*, 1990).

3.3.6 Natamycin uses in foods

The uses of natamycin as a preservative in foods and beverages are shown in Table 3.8. The main applications are for the surface treatment of cheeses and

Table 3.8 Applications of natamycin in foods and beverages, levels, method of addition and supporting references

Food application	Natamycin level (μg/g)	Method	References
Hard/semi-hard cheese	1250–2000	Surface treatment by spray or immersion	Delves-Broughton *et al.* (2006)
	500	Direct addition to coating emulsion	De Ruig and van den Berg (1985)
Grated cheese	15–20	Surface treatment by spray or direct addition	Berry (1999)
Meat products: dry sausage	1250–2000	Surface treatment by spray or immersion	Cattaneo *et al.* (1978), Caserio *et al.* (1974), Delves-Broughton *et al.* (2006)
Yoghurt	5–10	Direct addition to yoghurt mix	Şahan *et al.* (2004), El- Diasty *et al.* (2009)
Bakery products	1250–2000	Surface treatment by spray	Williams *et al.* (2005)
Tomato puree/paste	7.5	Direct addition	
Olives		Direct addition	Gourama *et al.* (1998)
Fruit juice, malt beverage	2.5–10	Direct addition	Shirk and Clark (1963)
Wine	30–40	Direct addition to stop fermentation	Thomas *et al.* (2005)
	3–10	Added prior to bottling to prevent secondary fermentation	

fermented sausages to prevent the growth of yeasts and moulds that are unsightly and can produce carcinogenic mycotoxins, and these two applications have wide regulatory approval. The three main methods of surface treatment of cheese are by spraying, dipping, or by applying the natamycin in a polyvinyl acetate (PVA) suspension coating. Fermented sausages are prone to mould spoilage during the ripening process as the pH drops and reduces the water holding capacity of the sausages, resulting in a decrease in moisture content and providing ideal conditions for the growth of yeasts and moulds. Use of natamycin for the surface treatment of cheeses and sausages is allowed in the EU and many other countries at a maximum level of 1 mg natamycin/dm^2 with a penetration depth of no more than 5 mm. In the USA, natamycin is not approved in meats but is approved in cheese at a maximum level of 20 μg/g, and also in other foods such as non-standardised yoghurt, cottage cheese, sour cream, non-standardised dressing, and marinades and sauces (Delves-Broughton *et al.*, 2005). Other existing or potential applications that have more limited authorisation are use on the surface of baked goods and in fruit juice, malt drinks, and wine. The application in wine is mainly in wines sweetened at the end of fermentation to prevent secondary fermentations from occurring (Thomas *et al.*, 2005).

3.3.7 Safety and tolerance

Natamycin was last extensively reviewed in 2003 by JECFA who confirmed that the previously established ADI of 0–0.3 mg/kg body weight was satisfactory and that consumption of treated cheese and meats would not exceed this ADI (www.inchem.org/documents/jecfa/jecmono/v48je06.htm). The EU have not set an ADI, hence use in the EU is restricted to the surface of cheeses and dried fermented sausages. The intravenous route is the path by which polyene macrolide antimicrobials are most toxic and oral administration is less toxic (Hamilton-Miller, 1973). There is apparently no adsorption of up to 500 mg/ day natamycin from the human intestinal tract after 7 days administration (Brik, 1981). Laboratory feeding studies to determine the above ADI were carried out by Levinskas *et al.* (1966) and are summarised by Delves-Broughton *et al.* (2005). Natamycin is used in the pharmaceutical industry for topical treatment of fungal infections of the eye and ring worm in horses and cattle.

3.4 Undefined fermentates used in food biopreservation

The use of spray-dried undefined fermentates produced by GRAS status lactic acid bacteria as culture organisms as a means of food preservation occurred in the USA with the introduction of MicroGARD® in the late 1980s and early 1990s (Weber and Broich, 1986; Ayres *et al.*, 1987, 1992, 1993). Since the original MicroGARD™ product was introduced various types aimed at specific target organisms have been marketed (Table 3.9). The important difference between these undefined fermentates and nisin and natamycin preparations are that they

Table 3.9 The MicroGARD® range of undefined microbial fermentates

MicroGARD® brand number	Composition	Target microorganism	Typical use level (%)	Application
100	Skim milk, cultured skim milk	Yeasts, moulds, Gram negative bacteria	0.1–1.5	Cottage cheese, sour cream, yoghurt, cultured dairy products, chocolate confections
200	Maltodextrin, cultured dextrose	Yeasts, moulds, Gram negative bacteria	0.1–1.5	Sauces, dressings, pasta
300	Skim milk, cultured skim milk	Lactic acid bacteria, Gram positive spore formers, *Listeria*	0.3–1.5	Some flavoured drinks
400	Skim milk, cultured skim milk	Yeasts, moulds, Gram negative bacteria, lactic acid bacteria	0.5–1.5	Various dairy products
520	maltodextrin, Cultured dextrose	Lactic acid bacteria, Gram positive spore formers, *Listeria*	0.25–1.5	Soups, salad dressings
730	Cultured dextrose, maltodextrin	Yeasts, moulds, Gram negative bacteria, lactic acid bacteria, Gram positive spore formers, *Listeria*	0.5–0.75	Cooked meat and poultry, refrigerated delicatessen salads
CM1–50	Cultured skim milk, maltodextrin	Gram positive bacteria	0.1–0.5	Dairy based products, dressings, prepared meals
CS1–50	Cultured dextrose, maltodextrin	Gram positive bacteria	0.1–0.5	Non-dairy based products, soups, sauces, dressings, prepared meals

are not purified by downstream processing so can be simply labelled as cultured milk or dextrose powder dependent on the fermentation substrate. As they are undefined their active ingredients are not declared. This in some countries, notably the USA, results in extremely friendly labelling when used in processed foods. They are simply declared as 'cultured skim milk' or 'cultured dextrose'. The EU, however, has decided not to adopt this approach and requires the labelling to declare the active ingredients contained. For this reason undefined fermentates are not used in the EU.

Various media can be cultured to produce the optimal concentration of antimicrobial metabolites. Also the media chosen can be similar to the final

application, such as 'cultured skim milk' for the dairy industry, 'cultured wheat' for the baking industry and 'cultured dextrose' for unrelated foods. The starters used in fermentate production are selected for their antimetabolite producing characteristics and frequently include lactic acid bacteria. Common within this group are the genera *Lactobacillus*, *Pediococcus*, *Propionibacterium*, *Leuconostoc* and *Lactococcus*. It should be noted that unpurified fermentates are not as active as purified fermenates such as nisin and natamycin preparations, so therefore end users usually need to use them at levels from 0.1% to as high as 1.5–2%. Any additional ingredient, particularly fermented products such as these, can impart an off-flavour. Antimicrobial activity must be balanced with organoleptic profiles when fermentates are used.

3.4.1 Physical, chemical and antimicrobial properties of fermentates

The physical, chemical and antimicrobial properties of microbial fermentates are as diverse as the cultures and media used to generate them. All are invariably combinations of mixed fermentation end products. Some of the most common commercially available fermentates available today, particularly with respect to total usage within the food industries, are based on the metabolites generated from the genera *Propionibacterium* and *Lactococcus* with either milk or dextrose used as the base starting media. Organic acids, obviously very common in lactic acid bacteria fermentates, usually contribute significantly to the chemical properties of end products. It is because of this that many of the fermentates are inherently very hygroscopic and will absorb moisture quickly in humid conditions. Consequently, they should always be kept in a cool dry environment.

In addition to rather high organic acid composition, there are always a number of known and unknown metabolites usually including, but not limited to, bacteriocins, enzymes, alcohols and small molecules that contribute to the overall physical and antimicrobial characteristics of the fermentate.

Fermentates, as their purified counterparts, are generally classified by which class of organism(s) they are designed to control, be they Gram negative bacteria, Gram positive bacteria, yeast and/or moulds. In some instances they can be multifunctional in having the ability to affect the outgrowth of more than one group of organisms. Likewise, blends of fermentates can be made which have a single label declaration (e.g. 'cultured dextrose'), but provide wide antimicrobial properties.

Propionibacteria are used in the manufacture of Swiss cheeses and also in the production of fermentates that are used frequently in the dairy and baking industries. Known as a source of organic acids, propionibacterial fermentates are able to supply these naturally generated, very heat stable antimycotics (Ray and Sandine, 1992). In general, propionibacterial metabolites have very little, if any, activity against Gram positive bacteria but do exert an inhibitory effect on many Gram negative bacteria. The modes of action against the latter are unclear but a number of published reports suggest that propionibacteria are capable of producing a variety of additional antimicrobial compounds against Gram negative

bacteria (Holo *et al.*, 2002; Van der Merwe, 2004; Grindsted and Barefoot, 1992; Gwiazdowska and Trojanowska, 2006; Ayres *et al.*, 1987; Al-Zoreky *et al.*, 1991). Because of activity against this class of organism, propionibacterial fermentates are widely used within the North American dairy industry to control the outgrowth of common spoilage organisms in fresh, cultured dairy products such as cottage cheese (Weber and Broich,1986; Ayres *et al.*, 1987, 1992, 1993).

Lactococci and pediococci form the bases of other commercially available fermentates. These have been formulated to interfere with the outgrowth of Gram positive bacteria. As with the propionibacteria, fermentates from these lactic acid bacteria contain significant amounts of organic acids in addition to small molecules and defined bacteriocins. Each specific fermentate possess its own antimicrobial characteristics.

Nevertheless, it should be kept in mind that all fermentates, because they are unpurified, possess antimicrobial activity that cannot be ascribed to a single molecule such as nisin, natamycin, pediocin or sakacin. Rather the activity is due to the cumulative effects of combinations of extracts, organic acids and various proteins and peptides. Assays for specific, single ingredients are invariably misleading as to the total activity present in the product and, consequently in the finished food.

3.4.2 Assay protocols and mode of action

It is imperative to reiterate that antimicrobial activity of fermentates cannot be ascribed to a single molecule. Consequently using biochemical analytical analyses (e.g. HPLC, GC, etc.) to determine the concentration of single components invariably generates misleading determinations. Optimal *in-vitro* inhibition assays are best done measuring total antimicrobial activity in the entire fermentate. On a routine basis, the agar diffusion methods of Tramer and Fowler (1964) and Fowler *et al.* (1975) are still used today. More recently turbidometric methods of Barreteau *et al.* (2004) and Turcotte *et al.* (2004) have been adopted for a more accurate and reproducible estimation of antimicrobial activity of fermentates.

Directly comparing *in-vitro* specific activity to that which would be expected *in-situ* is a common misconception with inexperienced users (Davidson and Branen, 2005). *In-vitro* assays are meant to monitor inhibition against specific organisms under precise growth conditions (medium composition, pH, temperature, etc.). In reality, the final results represent the net effects of microbial growth and antimicrobial inhibition. Results must be viewed as a careful balance between the two.

Because organic acids and their salts are routinely present in many commercially available fermentates, they invariably play a part in the overall inhibition spectra seen both *in-vitro* and in the finished foods. The modes of action of each of the organic acids present are unclear, but are commonly thought to be a function of the diffusion of a protonised (or undissociated) form of the molecule into the cell where the internal pH is lowered. In addition other factors may also be involved such as a disruption in active transport, nucleic acid replication and enzyme system integrity (Bogaert and Naidu, 2000).

The activity spectrum of a fermentate containing multiple organic acids can change dramatically depending on the environmental pH and individual dissociation constants. Understandably, it is extremely difficult to separate the antimicrobial contributions due to the contribution of organic acids mixtures from that of microbially generated bacteriocins. In essence all fermentates are component blends of known compounds together with those molecules we have some evidence do exist, but may be present in minute amounts.

3.4.3 Existing and potential uses in foods

Fermentates are used in a wide variety of refrigerated and ready-to-eat, minimally processed foods. In North America, simple propionibacterial based fermentates were first introduced into the dairy industry over 25 years ago to control the outgrowth of Gram negative bacteria, yeast and moulds in products such as cottage cheese, yogurt and sour cream (Salih *et al.*, 1990; Weber and Broich, 1986). Soon afterwards non-dairy versions ('cultured dextrose') found their way into products such as refrigerated soups, salad dressings, culinary items such as pasta fillings, prepared meals, side dishes and various cooked meat products. Likewise, propionibacterial fermentates were marketed heavily into the baking industry to 'naturally' control mould and rope spoilage. Available MicroGARD® products (Danisco) are shown in Table 3.9.

With the introduction of additional lactic acid based fermentates that target Gram positive bacteria, product applications for fermentate usage were expanded greatly. In addition to controlling spoilage contaminants, label friendly fermentates were also shown to be effective in controlling the outgrowth of certain pathogens such as *L. monocytogenes* in or on processed meat and poultry. Consequently 'all purpose' fermentates have been formulated to include additional ingredients such as rosemary extract, lysozyme and sodium diacetate, which act as antimicrobial potentiators (Bender *et al.*, 2001; Ming *et al.*, 1997). Refrigerated deli salads, various cooked meat and poultry products and prepared meals are typical users of fermentate blends.

Manufacturers have an impetus to utilise fermentates as ingredients as they can very often provide an alternative to chemical preservatives, afford a friendly 'natural' label declaration, reduce returns and possibly even protect from pathogen outgrowth. Most recently there has been a significant interest in pathogen control in minimally processed foods, and currently Salmonella outbreaks seem to be in the forefront. However there are few 'natural' solutions to Gram negative bacteria and coliform control, and available 'natural' Gram negative fermentates are static in nature.

3.4.4 Safety and regulatory status

The regulatory status of fermentates differs with each country and can vary significantly. Consequently it should be emphasised that regional and local authorities should be consulted prior to considering the use of fermentates as

antimicrobial hurdles in foods. For example in Canada fermentates are generally regarded as food ingredients. However they may also fall under the Canadian Food Inspection Agency's (CFIA) definition of a novel food which includes considerations such as its composition, history of safe use, whether it causes the food to undergo a major change or whether it was manufactured using genetically modified organisms (http://www.inspection.gc.ca/english/fssa/fispoi/product/novbroche.shtml). A number of cultured milk or dextrose fermentates can be used, not as novel foods, but as CFIA approved ingredients.

In the EU there are some who feel that the antimicrobial inhibitors present in any fermentate must be identified. However, because of the inherent, complex composition of fermentates, not all of the antimicrobial components can be defined. Consequently, they are currently classified under existing legislation. By default they are most often classified as food additives with their known antimicrobial components associated with preservatives, many of them chemical, bearing an 'E' number. As a consequence many countries limit the usage of fermentates to application areas where the preservatives are permitted.

In the United States there are a number of factors that must be considered in order for a fermentate to be Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA). Currently the most common method of FDA acceptance of a fermentate for food use is through the use of scientific procedures that utilise a panel of experts testifying both that the fermentate ingredients, cultures and their associated by-products have a history of safe consumption and that the naturally produced antimicrobial components have not been selectively purified or concentrated (http://www.fda.gov). The FDA and the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) have recognised a number of fermentates, including cultured skim milk or cultured dextrose, to be acceptable in a variety of foods products including meat and poultry products (http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/default.htm and http://www.fsis.usda.gov/Regulations_&_Policies/7000_Series Processed_Products/index.asp).

3.5 Future trends

There continues to be customer demand for minimally processed foods with a long shelf life that contain few, if any, chemical preservatives. At the same time there are also concerns about the high level of salt in our diet, with recommendations being made to reduce our intake. In many instances salt can be a major microbiological hurdle and reducing its level will have microbiological consequences both in terms of product safety and shelf life. Consequently, technology based on non-thermal treatment methods such as high pressure, pulsed electric field technology and active packaging systems will ensure that research and development into novel preservation systems will continue.

Likewise, specific antimicrobials such as purified and undefined fermentates as outlined in this chapter added to various non-thermal treatments are likely to play

an increased role. In this regard, control, regulation and harmonisation by foods safety authorities will be an important factor. The emergence of new microbiological problems and development of resistance will ensure that food technologists, molecular biologists and microbiologists will continue to search for new solutions.

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